High hydrostatic pressure pre-treatment of whey proteins enhances whey protein hydrolysate inhibition of oxidative stress and IL-8 secretion in intestinal epithelial cells

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Abstract

Background: High hyperbaric pressure treatment of whey protein isolate (WPI) causes changes in the protein structure that enhances the anti-oxidant and anti-inflammatory effects of WPI.
Objective: The aim of this study was to compare the anti-oxidant and anti-inflammatory effects of pressurized whey protein isolate (pWPI) vs. native WPI (nWPI) hydrolysates in Caco-2 cells exposed to hydrogen peroxide (H_2O_2).
Design: Cells were cultured with different concentrations of pWPI or nWPI hydrolysates either 1 h before or 1 h after H_2O_2. Cell viability, IL-8 secretion, intracellular reactive oxygen species (ROS), and the medium anti-oxidant capacity (FRAP assay) were measured.
Results: Prior to and after H_2O_2 exposure, pWPI and nWPI hydrolysates inhibited IL-8 secretion and ROS generation, and increased FRAP activity in a dose-dependent manner. The maximal inhibition of H_2O_2-induced IL-8 secretion was greater with 2000 μg mL^{-1} of pWPI (50%) vs. nWPI (30%) hydrolysates. At the latter concentration, inhibition of H_2O_2-induced ROS formation reached 76% for pWPI, which was greater than for nWPI hydrolysates (32.5%).
Conclusion: These results suggest that WPI hydrolysates can alleviate inflammation and oxidative stress in intestinal cells exposed to oxidative injury, which is further enhanced by hyperbaric pressure pre-treatment of WPI.

Keywords: *anti-oxidant*; *anti-inflammatory*; *reactive oxygen species*; *caco-2 cells*; *pressurized whey protein isolate hydrolysates*

Oxidative stress has been implicated as a potential etiological factor for inflammatory bowel disease (IBD) due to the well established detrimental effects of reactive oxygen species (ROS) in the gut inflammatory process (1). Oxidative stress can be caused by ROS such as hydrogen peroxide (H_2O_2), superoxide anion, singlet oxygen, or hydroxyl radicals (2) generated both in the lumen and in the intestinal mucosa (3). H_2O_2 is one of several ROS which causes oxidative stress that contributes towards colonic damage (2). H_2O_2 is generated via a variety of metabolic pathways and its intracellular accumulation can cause dysregulation of the normal metabolic processes partly by modifying proteins, lipids, and DNA (4). Also, H_2O_2 can activate transduction pathways such as mitogen-activated protein kinases that contribute to the production of pro-inflammatory cytokines such as (IL)-8 (5). The secretion of IL-8 is part of the immune response, particularly in inflammatory processes as IL-8 regulates the recruitment and activation of neutrophils in inflamed tissue (6). The dysregulation of IL-8 balance can lead to the type of serious tissue damage that has been reported in IBD (7) and in patients with

active colitis (8). Oxidative stress has been shown to promote IL-8 production in several cell types, including the human colon adenocarcinoma Caco-2 cell line (9), which has been widely used as an *in vitro* model of the intestinal epithelium (10). Exposure of Caco-2 cells to H_2O_2 can represent the pro-inflammatory response of intestinal cells following their exposure to oxidants generated in the lumen during digestion. Caco-2 cell cultures have been effectively used as an *in vitro* model for the investigation of toxicants, food mutagens, and their toxicological mechanisms affecting gut health (11, 12) and testing the protective action of anti-oxidants and nutraceutical agents (13, 14).

Bioactive proteins and peptides obtained from bovine milk proteins are being used as nutraceuticals to improve human health (14). Whey proteins, which comprise approximately 20% of the total proteins in cow's milk, have been found *in vitro* and *in vivo* studies to exert potent anti-oxidant and anti-inflammatory effects (15). Extensive disulfide linkages in whey proteins, however, increase their resistance to proteolysis and subsequent digestibility (16). The high resistance of the most prevalent whey protein, β-lactoglobulin (β-lg), to pepsin-mediated hydrolysis is considered to be a key factor that impedes whey protein digestibility (17, 18). Various studies have shown a low accessibility to peptide bonds localized to the interior of the globular structure of β-lg to the hydrolytic action of pepsin (19).

High hyperbaric pressure treatment of whey proteins can cause changes in their secondary and tertiary structure leading to denaturation (20). The most sensitive whey protein to denaturation induced by high hyperbaric pressure is β-lg, which contains two disulfide bonds and one free sulfhydryl group (21). Pressurization at 550 MPa has been demonstrated to increase the rate of β-lg hydrolysis due to partial unfolding of β-lg as the unfolded molecules are susceptible to proteolytic degradation (22). One study has shown that high hydrostatic pressure at 550 MPa causes a substantial increase in the rate of pepsin-mediated hydrolysis of whey protein isolates (WPI) (17). Cell culture, animal and human studies have indicated that hyperbaric pressure treatment of WPI increases their anti-oxidant and anti-inflammatory effects, which are linked to increased whey protein digestibility and the greater release of bioactive peptides (17, 23, 24).

A recent human trial has demonstrated that 1 month supplementation of pressure-treated WPI resulted in lowered inflammatory markers in patients with cystic fibrosis (25). Such supplementation has also been effective in countering gut associated inflammation as piglets with dextran sulfate-induced colitis fed pressure-processed WPI showed lower pro-inflammatory cytokine concentrations, decreased myeloperoxidase activity and improved antioxidant status in colonic tissue relative to both native WPI and skim milk protein-fed piglets (26).

Whey proteins could diminish gut tissue inflammation via effects exerted on macrophages as a recent study showed that hydrolysates of a formula containing 41% whey protein concentrate, along with a combination of vitamins and minerals, vegetable oils and inulin, down-regulated macrophage-mediated secretion of the pro-inflammatory cytokine IL-8 and tumor necrosis factor (TNF)-α in a co-culture with Caco-2 intestinal cells (27). The majority of peptides generated during the hydrolysis of whey proteins are not generally considered to be readily absorbed intact systemically. It is possible, however, that whey peptides could exert direct anti-oxidant and anti-inflammatory effects within intestinal cells since they have been shown to survive simulated human gastric, duodenal and jejuneal digestive processes (18). The capability of hydrolysates of native or pressure-treated WPI, however, to directly decrease oxidative stress and pro-inflammatory cytokine release within Caco-2 cells has not been previously examined. The present work therefore investigated the effects of hydrolysates of native and pressure-treated WPI on oxidative damage and inflammation induced by H_2O_2 in intestinal Caco-2 epithelial cells.

Methods

Whey protein isolate high hyperbaric pressure treatment and sample preparation

Whey protein isolate produced by membrane ultrafiltration was purchased (Vitalus Nutrition Inc., Abbotsford, BC, Canada). Whey protein isolate was pressurized in 12.5% (w/v) in double-distilled water and pressurized with an Avure High Pressure Processing System model QFP 215L-600 (Avure Technologies, Columbus, OH) by applying a single cycle of hyperbaric pressure of 550 MPa. This acts to unfold the protein conformation of the whey proteins enabling them to be more susceptible to digestive enzymes (17). The native whey protein isolate (nWPI) 12.5% (w/v) and the pressurized WPI (pWPI) 12.5% (w/v) WPI solutions were lyophilized using the Flexi-Dry MP Lyophilizer (FTS Systems, Stone Ridge, NY, USA) and freeze-dried under −80°C and 90 mTorr (mT) vacuum in preparation for future cell culture studies.

In vitro enzymatic digestion

The enzymatic digestion method was adapted from Vilela et al. (17) with some modifications (28). This technique mimicked the *in vivo* gastrointestinal digestion of milk proteins. Briefly, lyophilized native and pressurized WPI were diluted in 390 mL of distilled water to reach a final concentration of 3 mg protein mL^{-1} and the pH was adjusted to 1.9 with 12N HCl. The WPI digestion was started by adding freshly prepared pepsin (Sigma-Aldrich, Oakville, ON, Canada) solution to reach an enzyme:substrate (E:S) ratio of 1:200. Subsequently the pH was raised to 7.4 using 10N NaOH, which inactivated

pepsin irreversibly. After pepsin inactivation, the pancreatic enzymes trypsin (Sigma-Aldrich, Oakville, ON, Canada) and chymotrypsin Sigma-Aldrich, Oakville, ON, Canada) were dissolved in 10 mM NaH_2PO_4 to reach an E:S ratio of 1:200 and 1:87, respectively and immediately added to the solution. At the end of the digestion pH was increased to 10.5 to inactivate the pancreatic enzymes. The bottle was immediately chilled on ice under continuous stirring to decrease the temperature to 4°C.

Peptide isolation
After the digestion described above, the hydrolysates from native and pressurized WPI were subjected to an ultrafiltration system as described by Vilela et al. (17) and Iskandar (28). Briefly, to remove high molecular weight peptides a membrane filter of molecular weight cut-off (MWCO) of 10 kilodalton (kDa) (Millipore, Nepean, ON, Canada) was used in a stirred ultrafiltration membrane reactor (Model 8050, Millipore, Nepean, ON, Canada) under N_2 pressure of 40 psi. The major advantage to use this filter is to retrieve amino acids rich in leucine, tyrosine, phenylalanine, lysine, arginine, proline and aspartate (29). The filtrate was freeze-dried (Flexi-Dry MP Lyophilizer, FTS Systems, Stone Ridge, NY, USA) at −80°C and 90 mT vacuum for further cell culture experiments. The lyophilized filtrates were flushed under N_2 and kept at −20°C.

High-performance liquid chromatography (HPLC) profile
Reagents were HPLC grade and purchased from Sigma Chemical Co. (St Louis, MO, USA). Samples were analyzed using a Varian HPLC system with a tertiary gradient pump, a variable wavelength UV/VIS detector, and an autosampler with refrigerated sample compartment (Varian Canada Inc, Mississauga, ON, Canada). Samples were eluted using a Gemini-NX reverse-phase (RP)-HPLC column (100 ×4.5 mm) (Phenomenex, CA, USA), using a solvent flow rate of 1 mL min^{-1} and detection was at 215 nm. Gradient elution was carried out with a mixture of two solvents. Solvent A: 0.05% trifluoroacetic acid (TFA) in 10% aqueous acetonitrile (ACN) and solvent B: 0.05% TFA in 60% aqueous ACN, (v/v) starting with 100% solvent A and reaching 40% solvent A and 60% solvent B in 30 min.

Cell culture
The human colonic adenocarcinoma cell line Caco-2 was obtained from American Type Culture Collection (Manassas, VA, USA). All cell culture medium was obtained from Invitrogen (Carlsbad, CA, USA). Caco-2 cells passage numbers 21–29 were seeded at a density of 2.5×10^5 cells/well cultured in Dulbecco's modified Eagle's medium (DMEM, pH 7.4) with 25 mM glucose, supplemented with 20% heat inactivated fetal bovine serum (HFBS), 1% penicillin-streptomycin and 1% non-

essential amino acid solution in a humidified atmosphere containing 5% CO_2 at 37°C. All experiments were carried out in Minimal Essential Medium (MEM) with 2% HFBS and devoid of antibiotics.

Mitochondrial succinate dehydrogenase (MTS) assay
The effects of H_2O_2 on cell survival were measured using the MTS assay. Caco-2 cells were seeded in 24-well plates at a concentration of 2.5×10^5 cells/well. After 24 h of culturing, the cells were treated with different concentrations of H_2O_2 (0–2 mM) at 37°C for 1 h. Cells were then washed twice with MEM and incubated for 23 h. Subsequently, 10 μL of MTS reagent (Cayman Chemical, Ann Arbor, MI, USA) was added to each well and incubated for 3 h at 37°C. Finally, 100 μL per well of isopropanol containing 40 mM HCl was added to dissolve the formazan crystals. The absorbance was read at a wavelength of 560 nm using the Wallac Victor^2 1420 Series microplate reader (Harlow Scientific, Arlington, MA, USA). Treatments were compared with untreated control cells. Results are expressed as percentage of untreated control cells. Each point represents the mean of three experiments with each individual treatment being run in triplicate.

Enzyme-linked immunosorbent assay
Confluent Caco-2 cells on the 24-well plate were exposed for 1 h to a culture medium containing H_2O_2 (Sigma-Aldrich, Oakville, ON, Canada) ranging from 0 to 2 mM. After H_2O_2 was removed, cells were washed twice with MEM, and allowed to recover for 23 h. The supernatants were collected after 24 h of the treatment to determine IL-8 release using a commercially available ELISA kit (BD Biosciences, Mississaga, ON, Canada) according to the manufacturer's instructions.

Exposure of Caco-2 cells to WPI and H_2O_2
To investigate the protective effect of WPI, 0.25 mM H_2O_2-stimulated Caco-2 cells were exposed to different concentrations of nWPI or pWPI hydrolysates (0, 500, 1000 and 2000 μg mL^{-1}) in different 24-well plates. In this first set of experiments, Caco-2 cells were exposed to different concentrations of nWPI and pWPI hydrolysates for 1 h. WPI was removed, cells were washed twice with MEM, nWPI or pWPI hydrolysates were dissolved in MEM with 0.25 mM H_2O_2 and incubated for 1 h. Subsequently, Caco-2 cells were washed twice and nWPI or pWPI hydrolysates alone were added to the cells for 22 h. On the next day, supernatants were collected for IL-8 determination. A second set of experiments was performed to determine the recovery effect of nWPI or pWPI hydrolysates on pre-stimulated Caco-2 cells. Cells were exposed to 0.25 mM H_2O_2 for 1 h. H_2O_2 was removed, cells were washed twice with MEM, and nWPI or pWPI hydrolysates were added and cells

were allowed to recover for 23 h. The supernatants were collected for IL-8 determination 24 h after the treatment.

Ferric reducing anti-oxidant power (FRAP) assay

FRAP was used to determine the total anti-oxidant potential in the supernatant of Caco-2 cells exposed to 0.25 mM and different concentrations of nWPI or pWPI hydrolysates (0–2000 µg mL^{-1}). Briefly, in this assay the electron-donating capacity of the anti-oxidant was measured by the change in absorbance at 593 nm when a blue-colored Fe^{2+}-tripyridyltriazine (Fe^{2+}TPTZ) compound is formed from a colorless oxidized Fe^{3+} form. A standard curve was prepared from aqueous solutions of 1 mM $FeSO_4 \cdot 7H_2O$ at different concentrations ranging from 0.1 to 1.0 mM. The working reagent was prepared with 300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution dissolved in 40 mM HCl at 50°C, and 20 mM $FeCl_3 \cdot 6H_2O$ solution, at a 10:1:1 ratio, wrapped in foil. Once prepared it was immediately incubated for 10 min at 37°C. Thirty µL H_2O, 10 µL standards or samples, and 200 µL FRAP working solution were added in a 96 well plate. The reaction occurred at room temperature for 30 min and absorbance was taken at 593 nm in a microplate reader (Infinite PRO 200 series, Tecan Group, San Jose, CA, USA).

Intracellular reactive oxygen species (ROS)

The generation of intracellular ROS was evaluated by oxidation of 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA). The DCFH-DA was cleaved in the cells to dichlorofluorescin (DCFH) and oxidized by ROS to fluorescent dichlorofluorescein (DCF). This experiment explored the stimulation of the Caco-2 cells with 0.25 mM H_2O_2 for 1 h and after being washed twice, cells were treated with different concentrations of nWPI or pWPI hydrolysates from 0 to 2000 µg mL^{-1} for 23 h. After treatment cells were incubated with 5 µM DCFH-DA in phosphate buffered saline containing 5% (w/v) dimethyl sulfoxide (DMSO) solution for 30 min at 37°C. The fluorescent DCF was monitored by spectrofluorimetry (fluorescence excitation and emission were 485 nm and 530 nm, respectively; Wallac Victor^2 1420 Series spectrofluorimetry reader – Harlow Scientific, Arlington, MA, USA). The values were expressed as percentages of DCF fluorescence intensity to that of untreated control cells. Each point represents the mean of three experiments with each individual treatment being run in triplicate.

Statistics

Statistical analysis was performed using SAS for Windows (version 9.2, 2008, SAS Institute Inc., Cary, NC). One-way and two-way ANOVA were performed with Tukey's *post hoc* test to determine significant statistical differences between groups after verification of normality using Shapiro-Wilk.

Results

Effect of high hydrostatic pressure treatment on the peptide profiles of WPI hydrolysates

Figure 1 represents the peptide profiles of nWPI and pWPI with MWCO<10 kDa performed by HPLC analysis. Differences in the peak abundances as well as two new peaks were observed in the pWPI that were not present in the nWPI.

Effects of H_2O_2 cell viability and IL-8 response in stimulated Caco-2 cells

Cell viability as measured by the MTS assay following exposure of the cells to different concentrations of H_2O_2 is described in Fig. 2. The higher concentrations of H_2O_2 decreased ($P<0.05$) Caco-2 cells viability; however, the H_2O_2 concentration of 0.25 mM was associated with a similar number of viable cells as the control treatment. Disturbed integrity of the Caco-2 cell layer and detaching cells were also seen at treatment with 2 mM H_2O_2. Figure 3 represents the IL-8 response in Caco-2 cells exposed to different concentrations of H_2O_2. Increased ($P<0.05$) IL-8 concentrations relative to control Caco-2 cells was observed in cells treated with H_2O_2 concentrations from 0.25 to 2.0 mM with an approximate 300% increase observed with the lowest effective H_2O_2 concentration of 0.25 mM. The 0.25 mM H_2O_2 dose was used in subsequent experiments as it was not associated with decreased cell viability as opposed to the higher H_2O_2 concentrations (Fig. 2).

Effects of WPI hydrolysates on cell viability and inflammatory response

Figure 4 illustrates the effect of different concentrations of WPI hydrolysates on Caco-2 cell viability in the absence of H_2O_2. No cell toxicity was observed in cells treated with different concentrations of either nWPI or pWPI hydrolysates. The protective anti-inflammatory effects of nWPI and pWPI hydrolysates on H_2O_2-stimulated Caco-2 cells are shown in Fig. 5. Both types of WPI hydrolysates were associated with anti-inflammatory effects although pWPI hydrolysates showed lower ($P<0.05$) IL-8 release at each of the tested WPI hydrolysates doses. Stimulated Caco-2 cells treated with the highest dose of the hydrolysates of nWPI or pWPI of 2,000 µg mL^{-1} demonstrated a reduction ($P<0.05$) of IL-8 secretion of 26.3 and 37.7%, respectively, as compared with H_2O_2-stimulated Caco-2 cells alone. The Caco-2 cells stimulated with H_2O_2 had reduced ($P<0.05$) IL-8 concentrations when treated with either type of WPI hydrolysates for 1 h post-stimulation (Fig. 6). The inflammatory response in Caco-2 cells
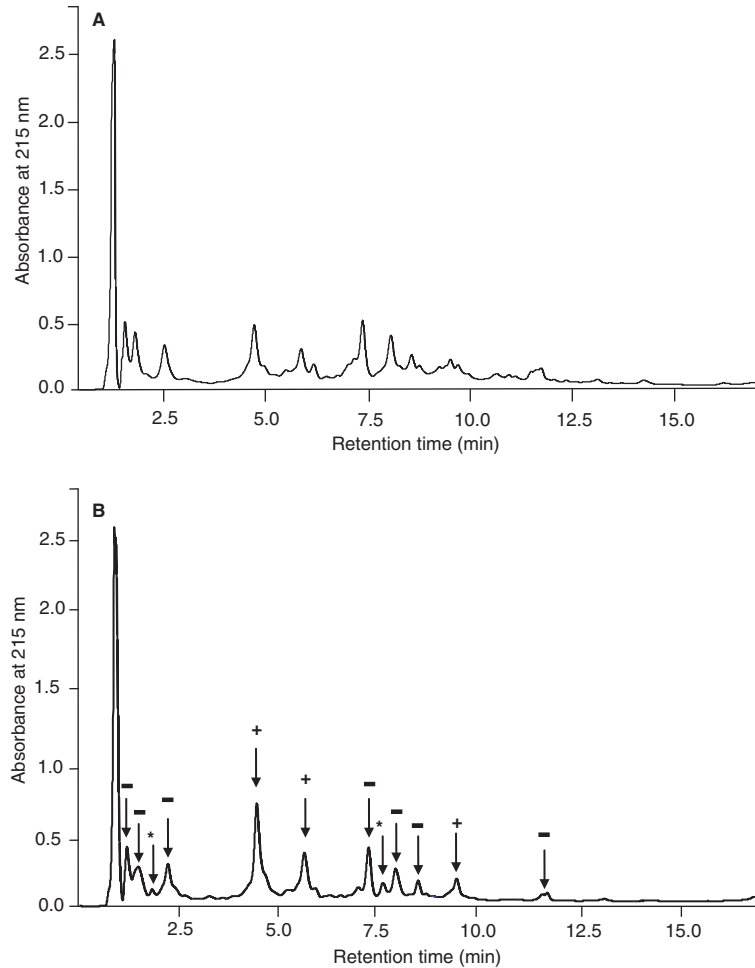
Fig. 1. Chromatogram of peptides from reverse-phase HPLC obtained via UV/VIS detector wavelength of 215 nm. (A) Peptides with MWCO less than 10 kDa derived from pepsin, trypsin and chymotrypsin hydrolysis of nWPI. (B) Peptides with MWCO <10 kDa derived from pepsin, trypsin and chymotrypsin hydrolysis of WPI exposed to hydrostatic pressure at 1-cycle of 550 MPa. The * symbol identifies extra peaks found in pWPH hydrolysates and arrows indicate differences in the relative abundances of peptides as a result of pressure treatment (+, higher abundance, −, lower abundance in pWPI hydrolysates relative to nWPI hydrolysates).
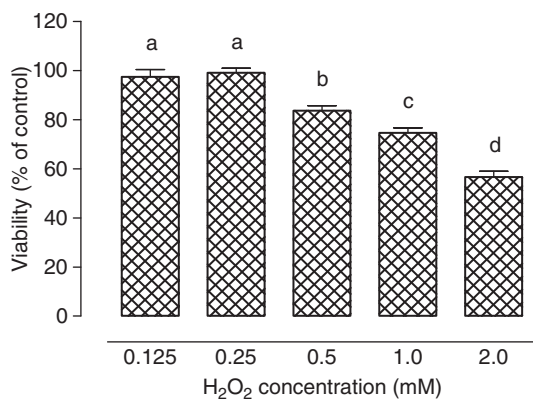
Fig. 2. Effects of different concentrations of H_2O_2 on Caco-2 cell viability. Values are means ± SE of three independent experiments. Means within each treatment without a common letter differ $P < 0.05$. H_2O_2 = Hydrogen peroxide.

induced by 0.25 mM H_2O_2 after 1 h post-stimulation treatment with the highest dose of nWPI or pWPI hydrolysates showed decreased ($P < 0.05$) IL-8 secretion of 30.4 and 50.1%, respectively.

Effects of WPI hydrolysates on the anti-oxidant capacity of cell culture medium

The ferric reducing anti-oxidant power (FRAP) measured in the cell culture medium of Caco-2 cells stimulated with 0.25 mM H_2O_2 after exposure to different concentrations of nWPI or pWPI hydrolysates is shown in Fig. 7. The medium of Caco-2 cells treated with either nWPI or pWPI hydrolysates showed increased ($P < 0.05$) in FRAP activity at all hydrolysates treatment concentrations. In addition, pWPI hydrolysates-treated Caco-2 cell culture media showed increased ($P < 0.05$) FRAP activity
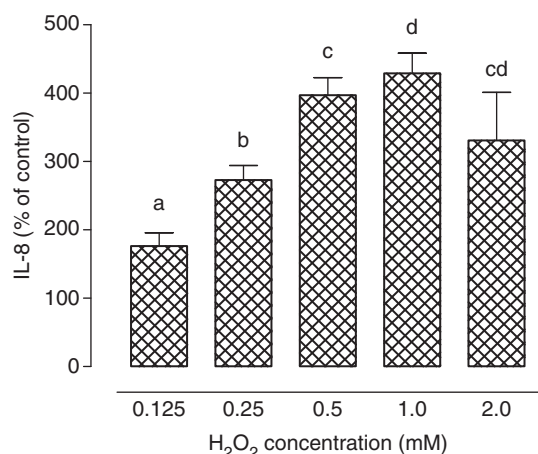
Fig. 3. Effect of different concentrations of H_2O_2 on IL-8 secretion of Caco-2 cells. Values are means±SE of three independent experiments. Means within each treatment without a common letter differ $P < 0.05$. $H_2O_2 =$ Hydrogen peroxide.

as compared to nWPI hydrolysates exposed Caco-2 cells at all tested concentrations.

Effects of WPI hydrolysates in the generation of reactive oxygen species (ROS)

The effects of different concentrations of WPI hydrolysates on Caco-2 cells exposed to 0.25 mM H_2O_2 are shown in Fig. 8. Caco-2 cells treated with either type of WPI hydrolysates showed a decrease ($P < 0.05$) in intracellular ROS production. Caco-2 cells treated with the highest dose of hydrolysates of nWPI or pWPI, i.e. 2000 µg/mL demonstrated a reduction ($P < 0.05$) in ROS formation of 32.5 and 76.1%, respectively, as compared with untreated Caco-2 cells. In addition, Caco-2 cells treated with pWPI hydrolysates had reduced ($P < 0.05$)
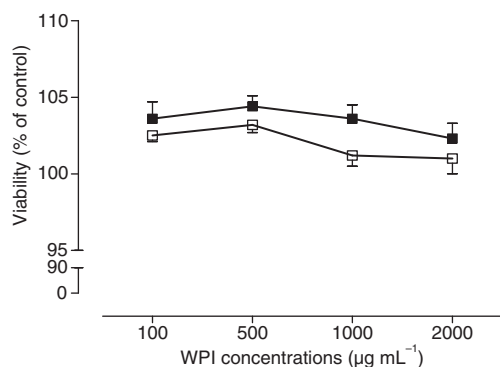

Fig. 4. Effects of different concentrations of WPI on Caco-2 cell viability. Values are means±SE of three independent experiments. Survival is expressed as percentage of untreated control cells. WPI=Whey protein isolate. (■) Native WPI, (□) Pressurized WPI.

ROS generation as compared to nWPI hydrolysates treated cells.

Discussion

The results of the present study have demonstrated that WPI hydrolysates can exert anti-oxidant and anti-inflammatory effects in H_2O_2-stimulated Caco-2 epithelial cells. The anti-oxidant effects of WPI hydrolysates as shown by decreased intracellular ROS generation and increased anti-oxidant capacity in the cell culture medium likely involved improved intracellular glutathione (GSH) status and enhanced anti-oxidant enzyme activities. Whey proteins are rich in the cysteine precursor required for synthesis of GSH, which acts as a major intracellular anti-oxidant partly by donating sulfhydryl protons to unstable ROS molecules (30). The reduced form of GSH is a substrate for GSH peroxidase that catalyzes the reduction of different peroxides such as H_2O_2 to H_2O (31). Previous work has shown that treatment with hydrolysates of pWPI was associated with improved intracellular status of the total and reduced form of GSH in cultured human lung epithelial cells (17). Caco-2 cells exposed to similar concentrations of H_2O_2 have shown attenuated intracellular oxidative stress when treated with the human whey protein lactoferrin (32); however, the present findings indicate that whey protein derived by-products of digestion such as peptides and amino acids can provide similar anti-oxidant protection.

The present study results showing an inhibited release of the pro-inflammatory cytokine IL-8 support the concept that hydrolysates of WPI can exert anti-inflammatory effects on intestinal Caco-2 cells exposed to oxidants (Fig. 5). IL-8 is the major cytokine measured in Caco-2 cells with respect to inflammation as compared to the inflammatory response assessed by the production of IL-6, prostaglandin E2, nitric oxide, IL-1β, TNF-α, and interferon-γ (33). IL-1β, an important mediator in intestinal inflammation, promotes IL-8 production (34). Oxidative stress induced via H_2O_2 has been shown to promote IL-8 production and increase IL-8 mRNA expression after H_2O_2-induced oxidative stress in Caco-2 epithelial cells (9). H_2O_2 up-regulates IL-8 gene expression via TNF receptors in the cell membrane, signaling the activation of the nuclear factor (NF)-κB pathway and IL-8 release (35). Our findings extend the results of a previous study showing that hydrolyzed whey protein formula exerted anti-inflammatory action on stimulated macrophages co-incubated with Caco-2 cells, which the authors attributed to synergistic effects of whey proteins and micronutrients present in the formula (27). The lowered IL-8 release in Caco-2 cell cultures shown in the present work could be related to the improved cellular redox status, which is closely regulated by the ratio of oxidized glutathione (GSSG) to the reduced form of GSH. Previous work has shown pretreatment of TNF-α stimulated
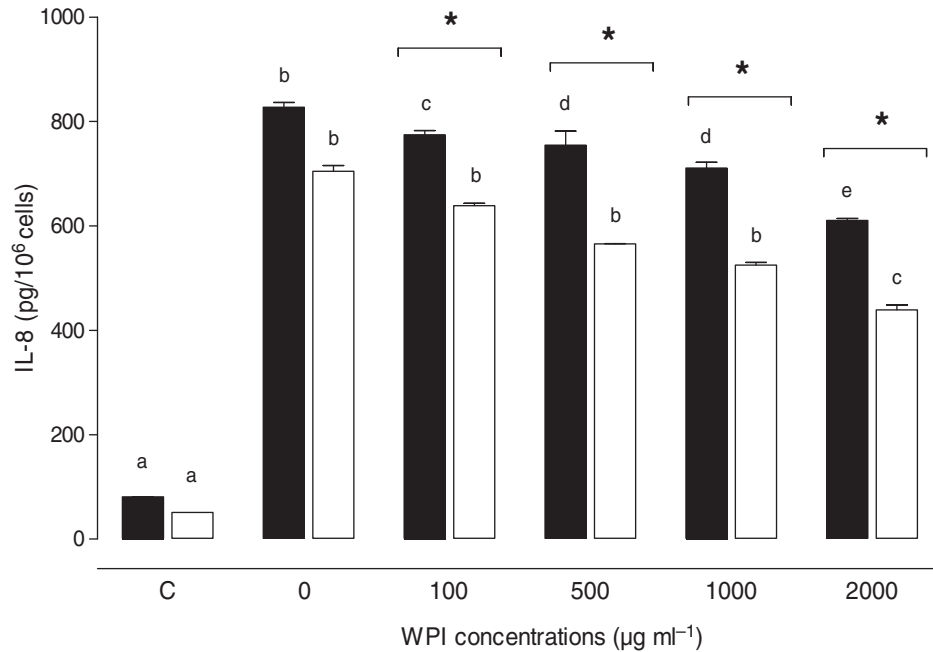
Fig. 5. Protective effect of different concentrations of WPI hydrolysates on 0.25 mM H$_2$O$_2$ induced IL-8 secretion by Caco-2 cells. Means within each type of WPI treatment without a common letter differ $P<0.05$. The * symbol represents the comparison between nWPI and pWPI treatments and significantly differ ($P<0.001$). C = Control. WPI = Whey protein isolate. (■) Native WPI, (□) Pressurized WPI.

human lung epithelial cells with hydrolysates of pWPI resulted in an improved cellular redox status that was related to decreased IL-8 secretion (17). When cells are exposed to increased levels of oxidative stress, GSSG accumulates and the ratio of GSH to GSSG decreases (36). A low GSH/GSSG ratio is linked with elevated levels
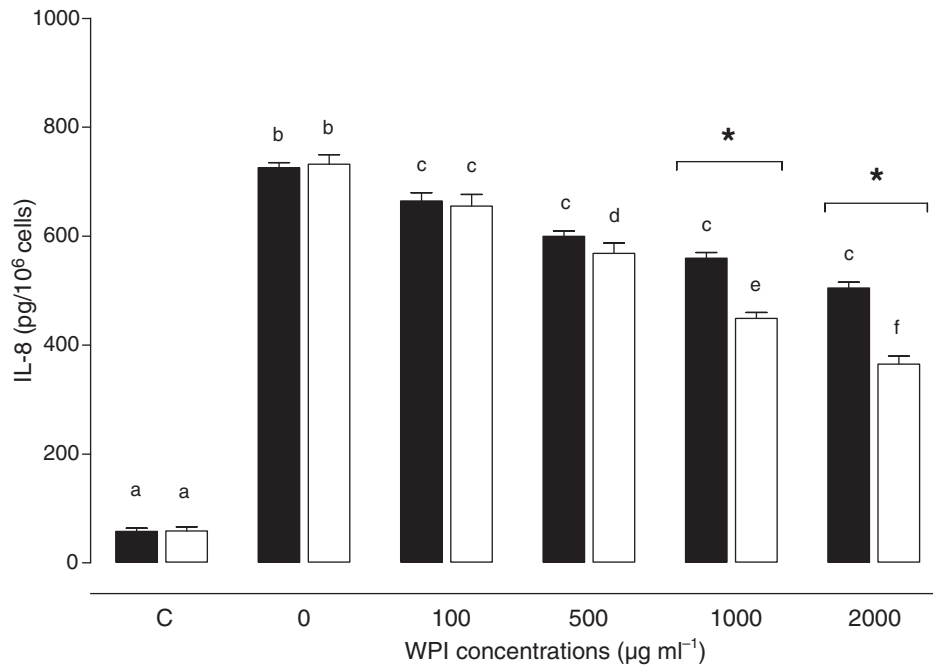

Fig. 6. Recovery effects of different WPI hydrolysates treatment with pre-stimulation of 0.25 mM H$_2$O$_2$ on IL-8 secretion of Caco-2 cells. Values are means ±SE of 3 independent experiments. Means within each type of WPI treatment without a common letter differ $P<0.05$. The * symbol represents the comparison between nWPI and pWPI treatments and significantly differ ($P<0.001$) C = Control. WPI = Whey protein isolate. (■) Native WPI, (□) Pressurized WPI.
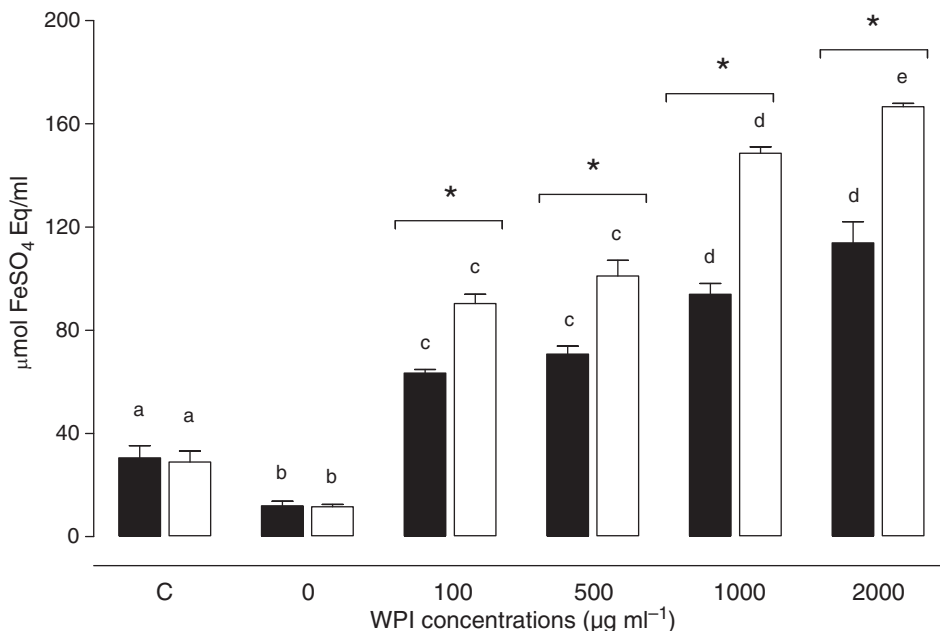
of pro-inflammatory cytokines since redox status is a key regulator of the inflammatory response (37). Apart from direct stimulation of GSH synthesis induced by precursor thiol-containing peptides and amino acids, other endogenous antioxidant pathways may be stimulated by the pWPI hydrolysates. For example, whey protein treated C_2C_{12} myoblasts showed activation of the transcription

Fig. 8. Effects of different WPI hydrolysates treatment on 0.25 mM H_2O_2-induced intracellular ROS generation in Caco-2 cells. Values are means$\pm$SE of three independent experiments. Means within each type of WPI treatment without a common letter differ $P<0.05$. The * symbol represents the comparison between nWPI and pWPI treatments and significantly differ ($P<0.01$). WPI =Whey protein isolate. (■) Native WPI, (□) Pressurized WPI.

factor NF-E2-related factor 2 that upregulates synthesis of endogenous antioxidant proteins and enzymes (38).

The anti-oxidant and anti-inflammatory effects of the WPI hydrolysates were enhanced by hyperbaric pretreatment of WPI. These findings are in agreement with recent observations that piglets with dextran sulfate sodium-induced colitis fed pressure-processed WPI showed significantly improved anti-oxidant and anti-inflammatory effects in the colonic tissue in comparison to native WPI and skim milk fed piglets, including decreased colonic content of IL-8, TNF-α and IL-18 (26). Similarly, hydrolysates of pressure-treated WPI exerted more effective anti-inflammatory effects than native WPI hydrolysates in stimulated cystic fibrosis transmembrane conductance regulator mutant respiratory cells (17, 39). Different peptide profiles were noted in hydrolysates resulting from the digestion of pressurized WPI including two new peaks (Fig. 1), which may have contributed to the greater anti-oxidant and anti-inflammatory effects of pWPI. Changes in the secondary and tertiary structures of pressure-processed whey proteins generates a unique profile of bioactive amino acids and peptides that enhances their anti-oxidant and anti-inflammatory effects (17, 23, 24, 26). In this manner, pressure processing of WPI might be comparable to the fermentation of whey proteins that has been shown to enhance the tissue antioxidant effects of whey proteins, which was related to an increased generation of the low molecular weight peptide γ-glutamylcysteine that can induce tissue GSH (40). Our previous studies have shown an increase in free sulfhydryl
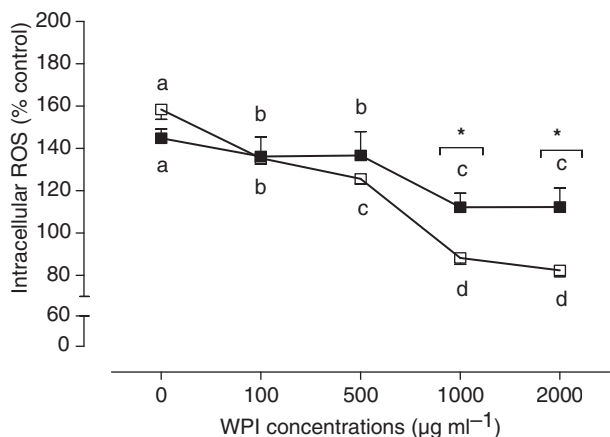
group content in WPI after high hydrostatic pressure treatment that we attributed to sulphide-disulphide interchange reactions (41). The antioxidant impact of an increase in free sulfhydryl groups in WPI is uncertain, however, as thiol-containing peptides may form aggregates via disulphide bond formation due to the high reactivity of thiol groups (42). Hydrolysates of pressurized WPI have also been shown to contain relatively higher free amino acid levels of alanine, histidine, glutamine, tryptophan, lysine and branched chain amino acids (BCAA) (17). It is conceivable that the combined effects of the above amino acids could have also contributed to the greater anti-inflammatory and anti-oxidant effects associated with pWPI since pre-treatment with those amino acids was shown to inhibit IL-8 secretion in H_2O_2-stimulated Caco-2 cells (39). Treatment with the BCAA valine, isoleucine, and leucine was also associated with elevated activities of both GSH S-transferase (GST) and catalase whereas histidine, tryptophan and lysine caused increase in GST activity (39). GST has ROS scavenging activity including the breakdown of lipid hydroperoxides (43) whereas catalase is involved in the decomposition of H_2O_2 (44). Glutamine has been reported to exert anti-oxidant effects in the Caco-2 and HCT-8 cell lines exposed to H_2O_2 by preserving cell membrane integrity and viability (45).

Conclusion

The findings from the present study have demonstrated anti-oxidant effects of hydrolysates of WPI in H_2O_2-treated Caco-2 cells that are improved with prior high hydrostatic pressure treatment of WPI. Furthermore, this study demonstrates an enhancement of the anti-inflammatory effects of WPI hydrolysates following by pressurization of WPI. More research is needed to measure a broader array of pro-inflammatory and anti-inflammatory cytokines to assess the extent of anti-inflammatory action associated with WPI hydrolysates. The above results, however, are consistent with the concept that whey hydrolysis products can act on intestinal cells directly to counteract oxidative stress and inflammation and so decrease tissue damage associated with inflammatory bowel conditions such as IBD. Our findings also indicate that the anti-inflammatory action associated with pWPI treatment in the piglet model of IBD (26) could be partly mediated via direct protective effects exerted on stimulated intestinal cells. Further studies are needed to investigate the specific native and pressurized WPI hydrolysate components involved in the protective effects as well as the mechanisms of action, which could involve down-regulation of the NF-κB pathway.

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